

## Research Article

**Growth kinetics of a *Helianthus annuus* and a *Salvia fruticosa* suspension cell line: Shake flask cultivations with online monitoring system**

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**Katja Geipel<sup>1</sup>****Maria Lisa Socher<sup>1</sup>****Christiane Haas<sup>1</sup>****Thomas Bley<sup>1</sup>****Juliane Steingroewer<sup>1</sup>**

<sup>1</sup>Institute of Food Technology and Bioprocess Engineering, Technische Universität Dresden, Dresden, Germany

**Correspondence:** Katja Geipel ([katja.geipel@tu-dresden.de](mailto:katja.geipel@tu-dresden.de)), Chair of Bioprocess Engineering, Faculty of Mechanical Science and Engineering, Institute of Food Technology and Bioprocess Engineering, Technische Universität Dresden, Bergstraße 120, 01069 Dresden, Germany.

## **Abstract**

Plants produce a variety of secondary metabolites, e.g. to defend themselves against herbivores or to attract pollinating insects. Plant cell biotechnology offers excellent opportunities in order to use such secondary plant metabolites to produce goods with consistent quality and quantity throughout the year, and therefore to act independently from biotic and abiotic environmental factors.

This article presents results of an extensive study of plant cell in vitro cultivation in a modern shake flask system with non-invasive online respiration activity monitoring unit. Comprehensive screening experiments confirm the successful transfer of a model culture (sunflower suspension) into the shake flask monitoring device and the suitability of this respiration activity monitoring unit as qualified tool for screening of plant in vitro cultures (sunflower and sage suspension).

The authors demonstrate deviations between online and offline data due to varying water evaporation from different culture flask types. The influence of evaporation on growth-specific parameters thereby rises with increasing cultivation time. Furthermore, possibilities to minimize the impact of evaporation, either by adjusting the inlet air moisture or by measuring the evaporation in combination with an appropriate correction of the measured growth values, are shown.

## **Practical applications**

Plant derived bioactive substances have been used in pharmacy and cosmetics for centuries, e.g. due to anti-inflammatory and anti-oxidative effects. However, it is still difficult and time-consuming to investigate plant cell culture behavior and optimize metabolite production using conventional agricultural methods.

Published results from monitoring experiments of sunflower and sage suspension cultures can be adapted in plant cell biotechnology for several cultivation systems. In addition, we confirm the practicability of the Respiration Activity MOnitoring System® for in vitro cultivation of plant cells. In this context, advantages and disadvantages of the RAMOS® device are discussed. Furthermore, we highlight the problem of evaporation out of the culture vessels, which is an important issue in combination with plant in vitro systems due to long runtimes. This publication is intended to help scheduling evaporation effects during the cultivation, especially according to flask neck wide and sterile closure type, and to prevent this aspect.

## **Keywords**

- Plant cell biotechnology
- Suspension culture
- Respiration activity
- Oxygen transfer rate
- Shake flask cultivation

## Abbreviations

CT	carbon dioxide transfer in $\frac{\text{mol}}{\text{l}}$
CTR	carbon dioxide transfer rate in $\frac{\text{mol}}{\text{l} \cdot \text{h}}$
LS	Linsmaier-Skoog (medium)
MS	Murashige-Skoog (medium)
OT	oxygen transfer in $\frac{\text{mol}}{\text{l}}$
OTR	oxygen transfer rate in $\frac{\text{mol}}{\text{l} \cdot \text{h}}$
OTR <sub>corr.</sub>	corrected oxygen transfer rate in $\frac{\text{mol}}{\text{l} \cdot \text{h}}$
R	universal gas constant in $\frac{\text{J}}{\text{mol} \cdot \text{K}}$
RAMOS	<u>R</u> espiration <u>A</u> ctivity <u>M</u> onitoring <u>S</u> ystem®
rpm	revolutions per minute
RQ	respiration quotient
t <sub>d</sub>	doubling time in d
V <sub>evap</sub>	evaporated volume in l
$\dot{V}_{\text{evap}}$	evaporated volume flow in $\frac{\text{l}}{\text{h}}$
V <sub>l</sub>	liquid filling volume in l
V <sub>t</sub>	total flask volume in l
x	dry biomass concentration in $\frac{\text{g}}{\text{l}}$
x <sub>max</sub>	maximum dry biomass concentration $\frac{\text{g}}{\text{l}}$
μ	specific growth rate in d <sup>-1</sup>
μ <sub>max</sub>	maximum specific growth rate in d <sup>-1</sup>
Δp <sub>O<sub>2</sub></sub>	oxygen partial pressure difference in bar
Δp <sub>CO<sub>2</sub></sub>	carbon dioxide partial pressure difference in bar
Δt	measuring time interval in h
2,4-D	2,4-Dichlorophenoxyacetic acid

## **1 Introduction**

### **1.1 Plant cell biotechnology**

Plants provide a huge variety of highly interesting bioactive substances which have been used by humans for centuries. They have been applied in pharmacy and cosmetics for example because of their anti-oxidative, anti-inflammatory, anti-microbial and fungicide effects [1, 2].

But the ever-growing world population increasingly demands more effective and less expensive methods for the supply with bioactive plant ingredients. To serve this growing request the field of plant cell biotechnology offers a promising alternative to conventional drug synthesis or extraction from agricultural grown plants [3]. With the help of plant in vitro systems cultivated in bioreactors, a production independent from biotic and abiotic factors is possible. Resulting metabolites are available all year in constant quality and quantity [4], and the yields are often higher than in the plant of origin [5, 6]. In addition, there are more opportunities to influence product yield (e.g. genetic modification, exclusion of pathogens or targeted increase of secondary metabolism) with less impact on nature or climate. Furthermore, product isolation from in vitro systems is easier than extraction from the complex matrix of an entire plant containing many different cell types due to organ structure [7].

The formation of these bioactive substances is basically separated from growth and division, but occurs in periods of low growth rates and often even under conditions of significant physiological or biochemical stress [8]. This stress-induced synthesis scheme, called elicitation is well applicable in plant cell biotechnology to increase product yield.

For the work described, callus or suspension cultures, respectively, are the plant in vitro culture types of interest. The totipotent callus acts as wound closure tissue and consists of undifferentiated cells [9 - 11]. It is naturally developed – controlled by phytohormones – to heal plant injuries and can be induced for biotechnological applications by the use of auxins and/or cytokinins with specific amount and composition [12]. Grown for maintenance on solid plant medium (containing agar), callus can be suspended in liquid plant medium (suspension culture) and, subsequently used for production processes in different types of bioreactors.

## 1.2 Biotechnological aspects concerning sunflower cell cultures

The annual sunflower (*Helianthus annuus*, Asteraceae) is well known as production system for  $\alpha$ -tocopherol, the most active form of vitamin E [12 - 14]. The group of vitamin E comprises lipid-soluble antioxidants, which are synthesised only by chloroplasts-containing organisms [15]. Due to their enormous potential to capture free radicals and to protect cells from oxidative damage [16], tocopherols are used in various industrial applications e.g. food-, cosmetic conservation [17 - 19]. Nowadays, most tocopherols are industrially produced on basis of chemical instead of biological syntheses, resulting in a reduction of vitamin-activity due to a racemic mixture of different and less effective stereoisomers. Only the all-R-form of  $\alpha$ -tocopherol – which can be obtained in high concentrations exclusive from plant products or parts [20, 21] – has the desired high biological activity [13, 22].

To gain more detailed information about plant in vitro systems, e.g. concerning plant cell physiology and product synthesis, several biotechnological studies deal with sunflower cell lines as model cultures using different bioreactor systems. Achieved biomasses and growth rates are summarized in table 1.

First considerable growth results for a shake flask cultivation of *Helianthus annuus* was presented by Scragg in 1990 achieving  $9.8 \frac{\text{g}}{\text{l}}$  dry biomass concentration and a growth rate of  $0.42 \text{ d}^{-1}$  [23]. In 1996, Kratchanova et al. reached a maximum dry biomass concentration of about  $15.0 \frac{\text{g}}{\text{l}}$  with a cultivation at same parameters [24]. Almost 10 years later Pavlov et al. and Haas published data of a sunflower suspension culture with a maximum dry biomass concentration of  $12.0 \frac{\text{g}}{\text{l}}$  [25, 26]. Data shown for cultivations in larger scale represent a similar picture: In 1990 Scragg published results for a 2 l flask. A maximum dry biomass concentration of  $11.3 \frac{\text{g}}{\text{l}}$  was achieved, resulting in a mean growth rate of  $0.32 \text{ d}^{-1}$ . In addition, the author presented a cultivation carried out in a 7 l air-lift bioreactor, wherein a maximum dry biomass concentration of  $10.3 \frac{\text{g}}{\text{l}}$  and a mean growth rate of  $0.29 \text{ d}^{-1}$  could be

reached [23]. A cultivation in a modified 5l-stirred tank reactor was performed by Haas et al. in 2008. The authors reported a maximum dry biomass concentration of about  $15.0 \frac{\text{g}}{\text{l}}$ . The maximum specific growth rate was estimated to  $0.20 \text{ d}^{-1}$  (ascertained between days 1 - 7) [27]. In summary, the data about growth-specific parameters of the cultivation of sunflower suspensions are very inhomogeneous, even in spite of similar cultivation parameters.

### **1.3 Shaking flasks with Monitoring System**

The Respiration Activity MOnitoring System® (RAMOS®, HiTecZang GmbH, Herzogenrath, Germany) combines the advantages of shake flasks and conventional bioreactors in terms of biotechnological aspects as it enables growth monitoring in small scale cultivation systems [28 - 31]. The RAMOS device is a shake flask system with eight measuring flasks, each equipped with an exhaust gas sensor. The recorded data of oxygen and carbon dioxide fractions in the gas phase can be used to calculate growth-dependent values like oxygen and carbon dioxide transfer (OT, CT), oxygen and carbon dioxide transfer rate (OTR, CTR) as well as the respiration quotient (RQ) at any time of the cultivation. Furthermore, the RAMOS device provides space for up to six reference flasks for sampling, which are standard Erlenmeyer flasks of the same size and geometry like the measuring flasks (here 250 ml). Compared to the manometric measuring method from Wen and Zhong published in 1995 [32], the RAMOS offers the ability to observe cell growth non-invasive and without changing the flow conditions in shake flasks [33].

## 2 Materials and Methods

### 2.1 Materials

#### 2.1.1 Plant Cell Culture

The experiments were performed with *Helianthus annuus* L. callus and a suspension culture of the same. The protocol for culture maintenance was adapted by Pavlov et al. [25].

#### 2.1.2 Medium and pre-culture

The callus culture was maintained on solid Linsmaier & Skoog (LS) medium (micro and macro elements including vitamins [34]), supplemented with  $30 \frac{\text{g}}{\text{l}}$  sucrose,  $0.2 \frac{\text{mg}}{\text{l}}$  2,4-Dichlorophenoxyacetic acid (2,4-D; solved in 96 % ethanol) and  $5.55 \frac{\text{g}}{\text{l}}$  phyto agar, with KOH (10 % (w/v)) adjusted to a pH of  $5.7 \pm 0.1$  prior to autoclaving. It was grown at 26 °C in the dark for 21 - 28 days.

The suspension culture was grown in the same LS medium without phyto agar. It was cultivated for maintenance in standard Erlenmeyer flasks (500 ml) with wide necks and wrapped paper plugs. Each flask was filled with 20 % (v/v) cell suspension composed of 20 % (v/v) of a 7-day old pre-culture and 80 % (v/v) LS medium, and cultivated on a shaker at 110 rpm (25 mm shaking diameter) and 26 °C in the dark for 7 days.

All media and material for cultivation were sterilized at 121 °C and 1 bar excess pressure for 15 min. All chemicals used for the media were purchased at DUCHEFA Biochemie B.V., The Netherlands.

### 2.2 Cultivation with the RAMOS device

#### 2.2.1 Inoculation

The inoculation was carried out with 20 % (v/v) pre-culture, 7 days old. But the small ports of the RAMOS measuring flasks as well as the aggregates formed by the plant cells (about 10 - 100 cells) represented a problem in terms of homogenous and reproducible inoculation. To solve this problem a “method of pre-inoculation” was used. Therefore the cell culture (20 % (v/v)) and LS medium (equates to a dry biomass concentration at the beginning of the experiment of approx.  $1.5 \frac{\text{g}}{\text{l}}$ ) needed



for all measuring and sampling flasks was mixed in advance in a separate sterile glass bottle. With the help of a sterilized glass funnel and a sterile measuring cylinder the inoculum was quickly separated in portions of 50 ml and transferred into the cultivating flasks; all steps were performed under aseptic conditions.

### **2.2.2 Cultivation system and parameters**

All flasks used in the experiments had a nominal volume of 250 ml. They were without baffles, made of glass, and the application of energy occurred by rotating (orbital movement). By default, all cultivations happened with 20 % (v/v) total filling volume. For an increase of reproducibility, all sterilized flasks have been dried prior usage. It is well known that "the wetting of the sterile closure ... may result in a significant reduction of the gas permeability of the plug" [35].

For maintenance the cells were cultivated in standard Erlenmeyer flasks with wide necks and wrapped paper plugs (Fig. 1A). A study to determine the evaporation characteristic of the setup was carried out using Erlenmeyer flasks with narrow necks and cotton wool plugs (Fig. 1B), in addition.

The cultivations for the determination of OT and CT as well as the rates OTR and CTR were performed with the RAMOS device in special measuring flasks (Fig. 1C), which are corresponding to 250 ml Erlenmeyer flasks concerning geometry in the lower two-thirds of the flasks. Furthermore, each RAMOS experiment was carried out with subsequent measuring and rinsing phases (40 min/50 min) for monitoring cell aeration. For a correct measurement, the flasks were automatically closed completely during each measuring phase followed by a flushing phase for aeration. These two phases alternated continuously. According to Büchs and Anderlei [28 - 30] the adjusted low aeration rate of  $10 \frac{\text{ml}}{\text{min}}$  per flask in the rinsing phase equates the indirect aeration through shaking of standard cultivation flasks. In accordance with Pavlov et al. the cultivations in the RAMOS were performed at 26 °C and 110 rpm (50 mm shaking diameter) in darkness [25]. The respiratory rates used for evaluation of cell growth were calculated according to equations (1) to (2) [30],

$$OTR = \frac{\Delta p_{O_2} * (V_t - V_l)}{\Delta t * R * T * V_l} \quad (1)$$

$$CTR = \frac{\Delta p_{CO_2} * (V_t - V_l)}{\Delta t * R * T * V_l} \quad (2)$$

with oxygen partial pressure difference  $\Delta p_{O_2}$ , carbon dioxide partial pressure difference  $\Delta p_{CO_2}$ , total flask volume  $V_t$ , liquid filling volume  $V_l$ , measuring time interval  $\Delta t$ , universal gas constant  $R$  and absolute temperature  $T$ . Furthermore, the equations (3) to (5) have been used to determine overall oxygen transfer  $OT$ , overall carbon dioxide transfer  $CT$  and respiration quotient  $RQ$ .

$$OT = \int_0^t OTR * dt \cong \sum_{i=0}^n \left( \frac{OTR_i + OTR_{i+1}}{2} * (t_{i+1} - t_i) \right) \quad (3)$$

$$CT = \int_0^t CTR * dt \quad (4)$$

$$RQ = \frac{CTR}{OTR} = \frac{\Delta p_{CO_2}}{\Delta p_{O_2}} = \frac{dp - dp_{O_2}}{-dp_{O_2}} \quad (5)$$

The included software HiBuilder (HiTec Zang, Herzogenrath, Germany) allows an automatic recording of the growth-related data.

### 2.2.3 Sampling and harvesting

For each experiment callus cells were cultivated in suspension for about 14 days. For evaluation of the online data, offline samples for determination of biomass, pH and conductivity were collected. Therefore, an entire sampling flask was harvested approx. every 48 hours.

### 2.2.4 Aeration with water-saturated air

To study the effects of aeration with water-saturated air on evaporation, a gas washing flask with a porous glass frit at the output was installed into the RAMOS device. This flask was filled with approx. 200 ml distilled water and connected to the inlet of the gas distributor for aeration of each measuring flask, ensuring an aeration

with defined and constant water-saturated air. The oxygen supply in the reference flasks was still carried out via indirect ventilation due to shaking (ambient air).

## **2.3 Analytical methods**

In order to determine the evaporated volume during cultivation, the whole sample was collected and subsequently refilled to the starting volume (50 ml) with distilled water. This step is important to receive correct data for conductivity and pH [33]. Subsequently, the cell suspension was filtered through a cotton tissue to separate biomass from supernatant.

### **2.3.1 Biomass determination**

The wet biomass was scraped from the filter on a previously dried and balanced petri dish. After weighing the fresh biomass in the petri dish, both were frozen (-20 °C) for at least 24 hours. Subsequently, the frozen biomass was freeze dried at -51 °C and 0.035 mbar. Petri dish and dried biomass were weighed to determine the amount of dry biomass. Furthermore, the calculation of specific growth rate  $\mu$  (Eq. 6), maximum specific growth rate  $\mu_{\max}$ , using the dry biomass concentration  $x$ , and the calculation of doubling time  $t_d$  (Eq. 7) were possible.

$$\mu(t) = \frac{\ln(x_2) - \ln(x_1)}{t_2 - t_1} \quad (6)$$

$$t_d = \frac{\ln 2}{\mu_{\max}} \quad (7)$$

### **2.3.2 Determination of medium components**

Conductivity and pH of the cell-free supernatant were measured at 25 °C.

### **2.3.3 Determination of evaporation behavior**

According to Mrotzek et al. [36] the new steady state method was used to ascertain the mass reduction due to evaporating water. Therefore, all cultivation flasks including sterile closures were weighed at intervals of approx. 24 hours.

### 3 Results and discussion

#### 3.1 Fundamental aspects of plant cell cultivations with the RAMOS device

The well-known differences between microbial and plant cells, e.g. in size, structure, and agglomeration tendency, result in numerous corresponding discrepancies regarding their in vitro cultivation (especially inoculation and sampling) [37, 38]. Based on first experiments (data not shown), the pre-inoculation was detected as the best approach for a reproducible culturing of plant suspensions in the RAMOS. So far, only few detailed experiments on the growth of plant cells in the RAMOS device have been published [31, 39, 40], and therefore preliminary studies on the adaptation of the plant cell lines to the system turned out to be required.

Figure 2 depicts online and offline data of the heterotrophic in vitro cultivation of a *Helianthus annuus* suspension in the RAMOS. The relative standard deviations ( $n=3$ ) for respiration quotient and oxygen transfer rate are on an average of 1 % (RQ) to 4 % (OTR) and a maximum of 6 % (RQ) to 16 % (OTR), which indicates the RAMOS to be a convenient measuring system for observation of plant cell growth. Furthermore, those small deviations imply that the pre-inoculation is a suitable method for reproducible inoculation with plant cells in suspension.

Regarding the gas transfer (OT, CT) in figure 2A, a classification into growth phases is possible, analogous to the growth stages of microorganisms: After a short adaption phase a change to exponential growth is detectable. Subsequently, due to the limitation on one or more nutrients, a linear growth phase seems to occur, leading to a turning point at  $t=90$  h. At this point, a limitation of oxygen is excluded. Experiments with varying filling volumes (10, 20, 30, 40 and 50 ml) did not reveal significant differences in the gas transfer rates (data not shown), as it would be expected in case of oxygen limitation. While the overall increase in biomass is visible until the end of the experiment (Fig. 2B), the sudden drop of the OTR curve at  $t=220$  h (after day 9 of the cultivation) indicates a transition to a phase with reduced respiration caused by limitation of an important nutrient e.g. sugar as carbon- and energy source [26, 30].

For comparison and to confirm the RAMOS as a good device for screening different cell lines, figure 3 shows the data of the cultivation of a *Salvia fruticosa* suspension

culture. Again, the growth status is easily detectable. Compared to the sunflower culture, this cell line has grown more slowly, which resulted e.g. in a turning point in the progress of OT from exponential to linear growth at  $t=135$  h (compared to  $t=90$  h for sunflower). Furthermore, a higher OTR maximum of  $3 \frac{\text{mmol}}{\text{l} \cdot \text{h}}$  is detectable in figure 3A.

For comparison of OTR values of plant in vitro systems with other biotechnologically attractive microorganisms, in 2001 and 2004 Anderlei et al. reported [29, 30] maximum oxygen transfer rates in the same cultivation system for the bacterium *Corynebacterium glutamicum* ( $18 - 39 \frac{\text{mmol}}{\text{l} \cdot \text{h}}$ ), the yeast *Saccharomyces cerevisiae* ( $20 \frac{\text{mmol}}{\text{l} \cdot \text{h}}$ ) and the fungus *Botrytis cinerea* ( $20 \frac{\text{mmol}}{\text{l} \cdot \text{h}}$ ), which are – in contrast to the presented data for plant cells ( $2 - 3 \frac{\text{mmol}}{\text{l} \cdot \text{h}}$ ) – about one order of magnitude higher. Main reason for this is the slower specific growth rate of plant cells. Additionally, when taking into account that an average plant cell is by a factor of 10 larger than a microbial cell [26], the reduced respiratory activity of plant cells can be explained by assuming a reduced total cell number per volume. Furthermore, Ullisch et al. refer to maximum oxygen transfer rates of about  $6 - 9 \frac{\text{mmol}}{\text{l} \cdot \text{h}}$  for virtually the same dry biomass concentration for a transgenic *Nicotiana tabacum* cell line [40]. *N. tabacum* is a relatively fast growing, well known, and therefore popular model system for plant in vitro studies [33, 39]. Reason for a higher respiratory activity of the *N. tabacum* in contrast to the *H. annuus* and *S. fruticosa* cell lines appear to be the different growth rates. In addition, differences in metabolism and/or genome characteristic which lead to higher oxygen demands are possible explanations [32].

To confirm the online data (Fig. 2A, 3A), the dry biomass concentration, pH and conductivity of the media were determined (Fig. 2B, 3B). Apart from the online device used, analysis and growth monitoring of plant in vitro cultures are difficult, e.g. due to thick suspension and formation of cell agglomerates. For example, the method of determining the optical density cannot be applied. For this reason in plant cell biotechnology a frequently used parameter to estimate cell growth is the conductivity

of the cell-free medium [25 - 27, 40]. Figures 2B and 3B ratify a good correlation between the decrease of ions in the medium and the increase of total cells mass.

A slow growth performance, typical for the comparatively large plant cells with complex metabolism [24 - 27], is noticeable (Fig. 2, 3). According to equation (6) as well as the online and offline data the maximum specific growth rate of the *H. annuus* cells could be calculated to be between 0.39 and 0.67 d<sup>-1</sup>. The computed doubling time (Eq. 7) for *H. annuus* on basis of the data in the exponential growth phase (day 1 - 4) is between 1.0 and 1.8 d (Tab. 2). This wide range of values is based on the use of different cultivation flask types. There are major differences between the estimated specific growth parameters from online (Tab.2, row 1) compared to offline data (Tab. 2, row 2 - 3). Further discrepancies are even within the offline data which is due to the known lower reproducibility of the offline methods. It is therefore important to remember that different types of culture flasks in terms of shaft sizes and sterile closures were used (Fig. 1). Thus, different evaporation behavior was assumed during the long culture time for plant cells of up to 14 days. It is worth mentioning, that according to Anderlei et al. and Raval et al. there is no difference in oxygen input into the culture broth between the flask types due to equal hydrodynamic conditions [29, 30, 39]. But, with regard to the evaporation characteristic and resulting viscosity increase, discrepancies are obvious [36, 42 - 44]. A further point is the different resolution of the two methods for calculation of growth-specific parameters (online versus offline): While the RAMOS collects 60 values in each measuring phase (approx. 1 value per minute, every 50 minutes), offline sampling only enables one value per 48 hours.

Compared to literature values for sunflower suspension cultures (Tab. 1;  $\mu_{\max}=0.21 - 0.42 \text{ d}^{-1}$ ), the maximum specific growth rates calculated from the dry biomass concentration ( $\mu_{\max}=0.39 \text{ and } 0.53 \text{ d}^{-1}$ ) are high but credible at least for the narrow flasks. In contrast,  $\mu_{\max}$  calculated from the online data (0.66/0.67 d<sup>-1</sup>) is up to three times higher, suggesting the measured OTR is greater than an oxygen demand, which is caused by pure biomass formation. The method for biomass determination has been validated prior (data not shown) and, by definition, the specific growth rate is calculated with the help of biomass values instead of respiration data (Eq. 6). The higher oxygen demand observed leads to the conclusion

that not the whole volume of oxygen supplied has been metabolized into biomass. For some reason there have to be other factors influencing the oxygen consumption (e.g. secondary and/or fermentative metabolism, storage of molecular oxygen) which can be also seen in the progress of the respiration quotient ( $RQ > 1$ ). Moreover, small fluctuations in the gas composition and moisture content of the inlet air due to the experimental setup cannot be excluded.

When comparing all data from experiments with literature values (Tab. 1, 2), it becomes obvious that the species-specific growth parameters are different and variable. The maximum specific growth rate, for example, is overall varying between 0.21 and 0.67 d<sup>-1</sup>, even at comparable cultivation regimes and identical conditions. Certain differences are due to the use of cell lines with different origins. But in general, this adverts to the difficulties of plant in vitro culturing especially concerning reproducibility. However, certain deviations in specific parameters are also known in the cultivation of microbial cells. The average maximum growth rates of microorganisms in contrast to the sunflower suspensions are greater by a factor of up to 120 and average doubling times are between 0.5 and 2.0 h. In 2006, Chmiel et al. reported values of 2.0 h<sup>-1</sup> for  $\mu_{\max}$  and 0.4 h for  $t_d$  for *Escherichia coli*, 1.5 h<sup>-1</sup> and 0.5 h for *Bacillus subtilis*, and 0.5 h<sup>-1</sup> and 1.5 h for *Saccharomyces cerevisiae*, each determined for cultivations under optimal conditions [45]. For this reason, plant cell biotechnology with the goal to produce secondary metabolites in large amounts is basically economic for slow-growing, woody plants; and if there is an advantage compared to microbial biosynthesis (e.g. complex biosynthetic pathways are not existing), chemical synthesis or traditional extraction processes, or if an alternative production route is absent [46].

### **3.2 Determination of evaporation in plant in vitro culturing with shaking flasks**

To get a deeper inside into the evaporation out of plant suspensions, a study was executed using standard Erlenmeyer flasks (Fig. 1A, B). The indirect aeration was carried out with ambient air with varying water content, e.g. depending on room temperature and air humidity. During cultivation, due to the long duration of an experiment with plant cells, evaporation has proven to be considerable. The calculation of the respiration rates through the RAMOS is – amongst others – based on the liquid filling volume in the flasks, whereby even at the end of the cultivation the

initial filling volume (in case of this issue 50 ml) is used as input for the equations (1) to (4). But when evaporation is not taken into account, significant errors can arise. For this reason plant cell cultivation experiments were executed including weighing all flasks in intervals of approx. 24 hours (new steady state method [36]). Moreover, comparative studies were performed on the evaporation characteristics in different flask types:

1. RAMOS flasks (Fig. 1C) at aeration with water-saturated air,
2. RAMOS flasks (Fig. 1C) at aeration with ambient air, as well as
3. reference flasks with wide necks and wrapped paper plugs (Fig. 1A), and
4. reference flasks with narrow necks and cotton plugs (Fig. 1B), type 3 and 4 at aeration with ambient air.

On basis of this evaporation study a model for correction of the OTR was developed. It should be noted that a humidification of inlet air was exclusively possible for the eight RAMOS measuring flasks. Furthermore, the relative humidity of ambient air is expected to be in the range of 30 to 60 %; it should be measured during future experiments.

To determine the entire evaporated liquid volume, it was assumed a constant density of the evaporated liquid of  $0.997 \frac{\text{g}}{\text{cm}^3}$  (valid for water at 26 °C). A summation (Tab. 3) results on an average of 2.3 ml evaporated liquid for a cultivation without defined humidification (ambient air with varying moisture content) for 7 days, and 0.5 ml evaporated water for a cultivation with humidification, when cultivating in RAMOS flasks. In the reference flasks, the in vitro cultivation of sunflower suspensions results in 6.8 ml volume loss in wide neck flasks with wrapped paper plugs (Fig. 1A) and 4.3 ml loss in narrow neck flasks with cotton wool plugs (Fig. 1B). The evaporation values mainly depend on temperature, viscosity and shaking frequency and are valid only for the cultivation at 26 °C and 110 rpm with the flask types mentioned.

The study confirms the expected variation in the evaporation behavior between the different shaft widths, sterile closures and moisture contents of inlet air. For reproducibility, the usage of a humidified aeration is desirable because in this case the water content in the inlet air is constant and the evaporation at a minimum (< 1 %



for RAMOS flasks). In this way, e.g. effects of accumulation of medium components and its (negative) impacts on cell culture can be avoided. In contrast, the evaporation in the standard cultivation flasks with wide necks is more than 10 times higher (> 10 %). At this point, the expected discrepancy with respect to evaporation between RAMOS and reference flasks is significant. But aeration at constant water-saturation is not part of the usual equipment of a standard shaking incubator and is thus difficult to realize for standard Erlenmeyer flasks. Another possibility is to refill the flasks in certain time intervals with distilled water. Thereby, however, effort and risk of sterility are quite high. The better option to ensure the comparability between offline values and online data from the RAMOS seems, to adjust the calculation of the respiration values based on existing evaporation data according to equation (8), CTR analogous.

$$\text{OTR}_{\text{corr.}} = \frac{\Delta p_{\text{O}_2} * (V_t - (V_{l,0} - \dot{V}_{\text{evap}} * t))}{\Delta t * R * T * (V_{l,0} - \dot{V}_{\text{evap}} * t)} \quad (8)$$

With help of the developed model it is possible to correct the transfer rates and further respiration values determined by the RAMOS: The initial liquid volume in the flask  $V_{l,0}$  is reduced by the previously determined, flask-type dependent volume of evaporation  $\dot{V}_{\text{evap}}$  (Tab. 3). However, this manner of adjustment of the OTR e.g. does not take into account effects by accumulation of medium components and thus altered growth conditions.

Figure 4A shows the mean courses of the evaporation of water from the culture broth for RAMOS compared to reference flasks. As expected, the evaporation rate increases with reduced water content in the inlet air (compare the progress for RAMOS flasks: filled and empty dots) and with increasing throat diameter of cultivation flasks. In the same direction a decrease of the coefficient of determination  $R^2$  for the linear model is detectable (Tab. 3). That means the error between real evaporation behavior and linear model is especially visible in the reference flasks. An adaptation of the model with the help of an exponential equation for example should be considered for further investigations.

Figure 4B shows the comparison of two curves of oxygen transfer rates of a cultivation in the RAMOS in measuring flasks with the standard aeration (aeration with ambient air): While one curve does not contain the mathematical correction for evaporation, the second is corrected according to equation (8). As expected, both curves separate increasingly during time due to incremental water loss. As the corrected graph runs above the non-corrected curve, evaporation results in a lower increase of the OTR during the growth phase. The deviations between the two curves are not very high (coefficient of variation  $< 1\%$  at  $t=24\text{ h}$  and  $< 10\%$  at  $t=240\text{ h}$ ), but it has to be considered that the presented OTR progress originates from a measuring flask with an appropriate small shaft width for evaporation.

The discrepancies between the respiration activities are expected to be even greater when comparing RAMOS measuring with reference flasks because of the major differences in evaporation behavior (Tab. 3) due to the different bottle necks and sterile closures (Fig. 1, 4A). To confirm this statement, it is necessary to measure respiration activity in the reference flasks for a comparison with data from the RAMOS. Subsequently, the detection of (different) growth behaviour depending on flask type, sterile closure and/or aeration moisture content is required. This could be content of a future study concerning evaporation kinetics. In the meantime, the equation for correction of the established OTR (Eq. 8) is provided to close the existing gap between the knowledge we have about respiration in a plant in vitro shake flask system and the offline data we get from a slightly different cultivation regime (measuring flask versus reference flask).

## 4 Concluding remarks

The results provided in this publication are part of a comprehensive investigation regarding the suitability of a modern respiration device for screening of plant in vitro cultures. First detailed screening experiments with sunflower and sage suspension cultures and related data, demonstrating the successful cultivation in the Respiration Activity MOnitoring device, are shown. This can be regarded as the first step of an optimization of cell line, medium and cultivation parameters in this small scale culturing system, followed by a production process for secondary plant metabolites in a larger scale.

The RAMOS proved to be an advanced tool for fast and parallel screening of plant in vitro cultures. Nevertheless, an important criterion which has to be considered was determined during broad investigations: The relationship between respiration activity and evaporation could be revealed and confirmed as important factor in the long cultivation period of plant cells (about 7 to 14 days). Depending on throat diameter and sterile closure material significant water losses arise, generating complications when comparing RAMOS online data with offline data from cultivations in standard Erlenmeyer flasks. To ensure reproducibility and comparability, flasks with narrow necks and cotton wool should be used for all cultivations in the RAMOS (at aeration with ambient air). As table 3 and figure 4A depict, the deviations between this two flask types are at a minimum. For adjustment of existing equations for growth-specific respiration parameters, a correction factor was introduced; in this article presented at the example of oxygen transfer rate OTR (Eq. 8).

For further investigations it is recommended to measure the relative humidity in the cultivation environment to investigate influences on evaporation behavior, e.g. day and night rhythms. In addition, the viscosity increase caused by the formation of exopolysaccharides, cell growth and water evaporation should be investigated as it leads to inhomogeneities, limitations and reduced production rates [47].

Moreover, in future studies the influence of the intermittent shaking motion, which is required to determine the evaporation or to take samples, should be considered due to limiting nutrient supply [35]. Summing up, the RAMOS offers a good and fast opportunity to screen for cultivation parameters such as medium, temperature,

shaking frequency as well as elicitation agents and can be successfully applied for investigations in plant biotechnology.

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## **Conflict of interest statement**

The authors have declared no conflict of interest.

## Tables

Table 1. Comparison of growth specific parameters for the cultivation of *H. annuus* suspension cultures in different bioreactor systems based on literature references.

Bioreactor system	Max. dry biomass [ $\frac{g}{l}$ ] (day of max.)	$\mu_{max}$ [ $d^{-1}$ ] (day of max. value)	$t_d$ [d]	Reference
250 ml shake flask	9.8 (day 8)	0.42 (mean)	1.65	[23] <sup>(2)</sup>
250 ml shake flask	15.0 (day 6)	0.215 <sup>(1)</sup>	3.224 <sup>(1)</sup>	[24]
250 ml shake flask	12.0 (day 9)	0.21	3.31	[25]
250 ml shake flask	16.1 (day 10)	0.26 (day 0-4)	2.66	[26]
2 l shake flask	11.3 (day 11)	0.32 (mean)	2.20	[23] <sup>(2)</sup>
5 l stirred tank reactor	13.0 (day 9)	0.21 (day 5-8)	3.30	Results from previous own work
5 l stirred tank reactor	15.2 (day 10)	0.20 (day 1-7)	3.46	[27]
	15.0 (day 10)	0.16 <sup>(1)</sup> (mean)	4.33 <sup>(1)</sup>	
7 l air-lift bioreactor	10.3 (day 7)	0.29 (mean)	2.40	[23] <sup>(3)</sup>
7 l air-lift bioreactor	-	0.205 <sup>(1)</sup>	3.381 <sup>(1)</sup>	[23] <sup>(3)</sup>
100 l air-lift bioreactor	9.5 (day 10)	0.30 (mean)	2.30	[23] <sup>(3)</sup>

(1) Manual calculation by the authors on basis of a biomass curve presented in the reference; for 20 % (v/v) inoculum.

(2) Medium and shaking frequency used in the reference differ from the parameters applied by the authors.

(3) Medium used in the reference differs from the medium applied by the authors.



Table 2. Summary of specific growth parameters for the cultivation of *H. annuus* suspension cultures in different shake flask types ( $V_f=250$  ml), calculated on the basis of online (OTR,  $n=7$ ) and offline (dry biomass concentration,  $n=5$ ) data.

Shake flask type	$x_{\max} [\frac{g}{l}]$ (day of maximum)	$\mu_{\max} [d^{-1}]$ (day of maximum)	$t_d [d]$	Basis of calculation
RAMOS (measuring flask, Fig. 1C)	-	0.66 (day 1)	1.05	Online value: OTR <sup>(4)</sup>
Erlenmeyer flask, narrow neck, cotton wool plug (ref. flask, Fig. 1B)	13.9 (day 11)	0.39 (day 4)	1.78	Offline value: dry biomass concentration <sup>(5)</sup>
Erlenmeyer flask, wide neck, wrapped paper plug (ref. flask, Fig. 1A)	8.2 (day 11)	0.53 (day 4)	1.31	Offline value: dry biomass concentration <sup>(5)</sup>

(1) Manual calculation according to equations 6 and 7.

(2) Manual calculation; calculation over the whole growth phase.

Table 3. Summary of the evaporation study for the cultivation of *H. annuus* suspension cultures in different shake flask types (26 °C, 110 rpm; 250 ml nominal flask volume, 50 ml initial filling volume; assumption: evaporation of water with constant density; equations for linear models: intersection with y-axis b=0). Maximum and minimum values are highlighted.

Type of flask	RAMOS flask (Fig. 1C)		Standard Erlenmeyer flask	
Characterisation of aeration and flask neck	Water-saturated air, special neck with cotton wool plugs	Ambient air, special neck with cotton wool plugs	Ambient air, narrow neck with cotton wool plugs (Fig. 1B)	Ambient air, wide neck with wrapped paper plugs (Fig. 1A)
Evaporation rate [ $\frac{\text{mg}}{\text{h}}$ ]	$2.79 \pm 0.21$	$13.93 \pm 1.50$	$25.43 \pm 1.98$	$40.57 \pm 8.20$
Loss of water during 7-day culturing: total vol., percentage of start vol.	0.47 ml, <b>0.94 %</b> (min.) (n=7)	2.34 ml, 4.68 % (n=16)	4.27 ml, 8.54 % (n=15)	6.82 ml, <b>13.63 %</b> (max.) (n=5)
Model equation	$V_{\text{evap}} = 0.0627 \frac{\text{ml}}{\text{d}} * t$	$V_{\text{evap}} = 0.3643 \frac{\text{ml}}{\text{d}} * t$	$V_{\text{evap}} = 0.6013 \frac{\text{ml}}{\text{d}} * t$	$V_{\text{evap}} = 1.1067 \frac{\text{ml}}{\text{d}} * t$
Coefficient of determination $R^2$	0.9858	0.9997	0.9895	0.9665

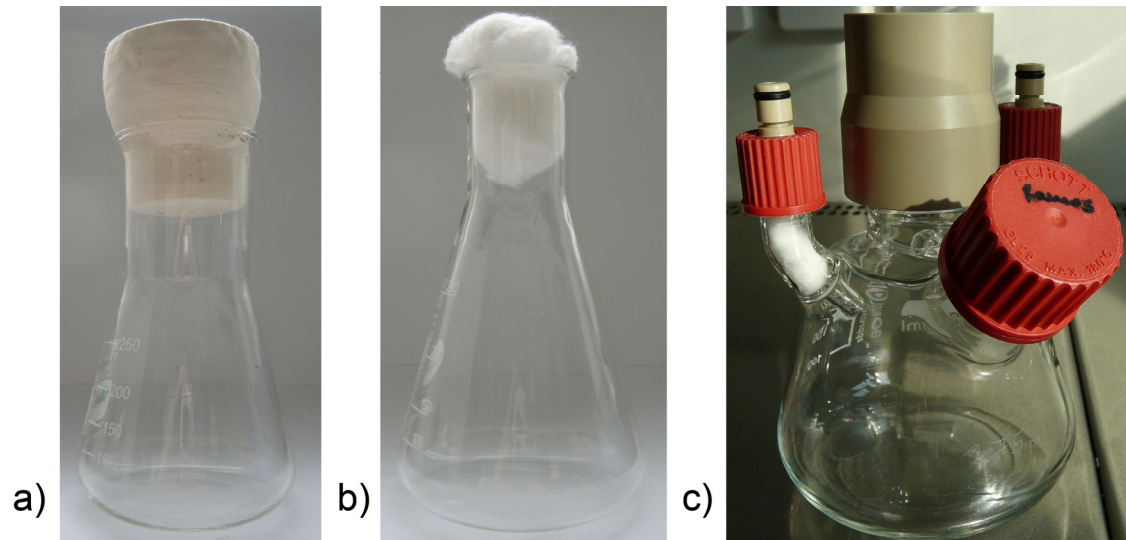


Figure 1. Flask types used for cultivation of *H. annuus* suspension cultures ( $V_t=250$  ml). **(A)** Reference flask with wide neck and wrapped paper plug. **(B)** Reference flask with narrow neck and cotton wool plug. **(C)** RAMOS flask.

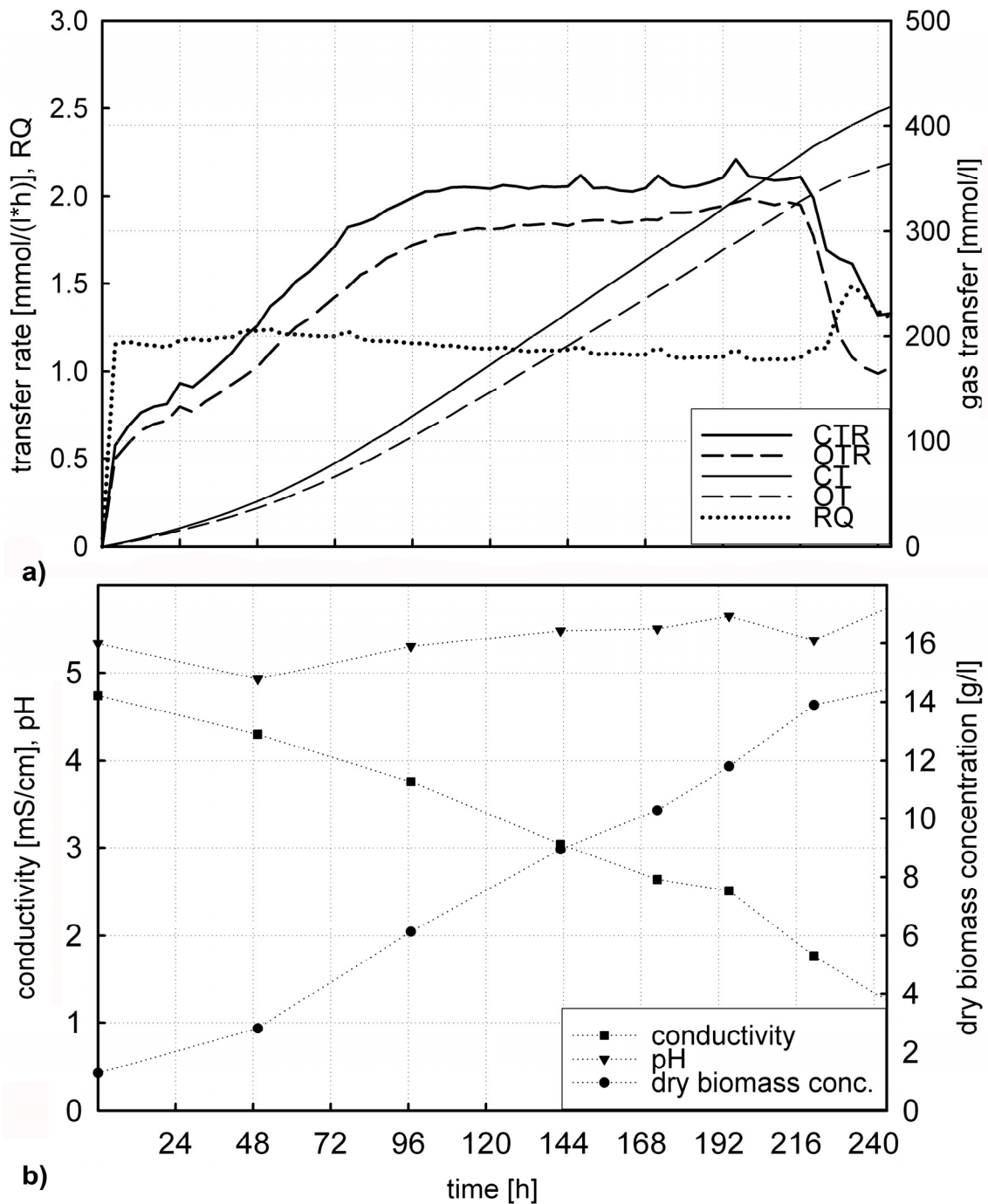


Figure 2. **(A)** CTR, OTR, CT, OT, RQ (n=3) of a *H. annuus* suspension culture in the RAMOS. **(B)** Offline values: conductivity, pH and dry biomass concentration (n=1). Cultivation conditions: rotary shaker, 110 rpm, 50 mm shaking diameter, 26 °C, 250 ml nominal flask volume, 50 ml initial filling volume, LS medium with 30  $\frac{\text{g}}{\text{l}}$  sucrose and 0.2  $\frac{\text{mg}}{\text{l}}$  2,4-D, pH 5.7; in darkness; aeration with ambient air.

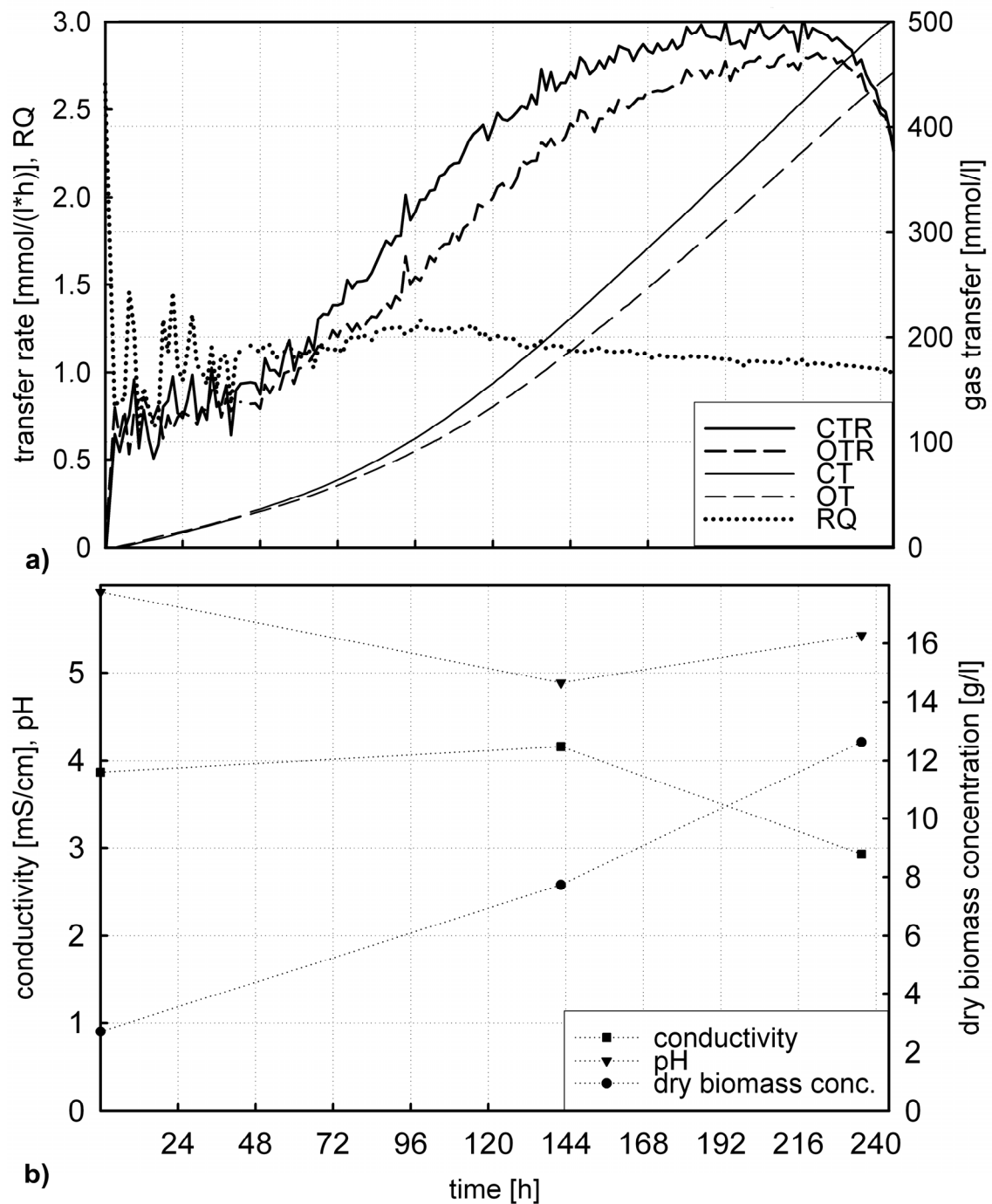


Figure 3. **(A)** CTR, OTR, CT, OT, RQ (n=4) of a *Salvia fruticosa* suspension culture in the RAMOS. **(B)** Offline values: conductivity, pH and dry biomass concentration (n=1). Cultivation conditions: rotary shaker, 110 rpm, 50 mm shaking diameter, 26 °C, 250 ml nominal flask volume, 50 ml initial filling volume, MS medium [41] with  $30 \frac{\text{g}}{\text{l}}$  sucrose,  $0.5 \frac{\text{mg}}{\text{l}}$  2,4-D and  $0.5 \frac{\text{mg}}{\text{l}}$  Zeatin, pH 5.7, in darkness, aeration with ambient air.

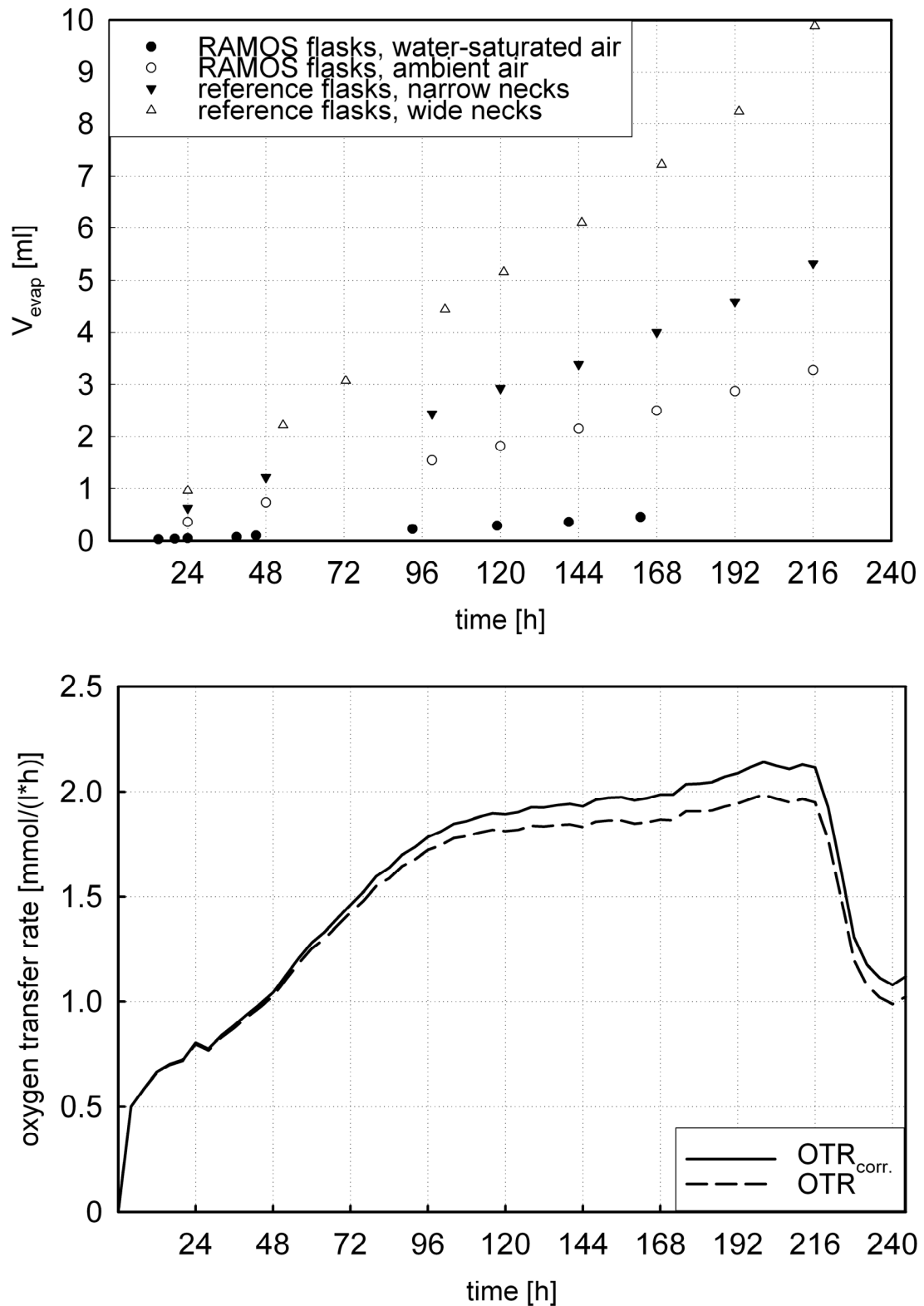


Figure 4. **(A)** Evaporated volume during cultivations of sunflower suspension cultures. Filled dots: RAMOS flasks with water-saturated aeration (n=7). Empty dots: RAMOS flasks with aeration using ambient air (n=7). Filled triangel: Reference flasks with narrow necks (n=6). Empty triangel: Reference flasks with wide necks (n=6). **(B)** OTR of cultivation of a *H. annuus* suspension culture in the RAMOS. Dashed line:

calculated without regarding evaporation (n=3). Straight line: evaporation-corrected according Eq. 8 (n=3). Cultivation conditions: rotary shaker, 110 rpm, 50 mm shaking diameter, 26 °C, 250 ml total flask volume, 50 ml initial filling volume, LS medium with  $30 \frac{\text{g}}{\text{l}}$  sucrose and  $0.2 \frac{\text{mg}}{\text{l}}$  2,4-D, pH 5.7, in darkness, aeration with ambient air.